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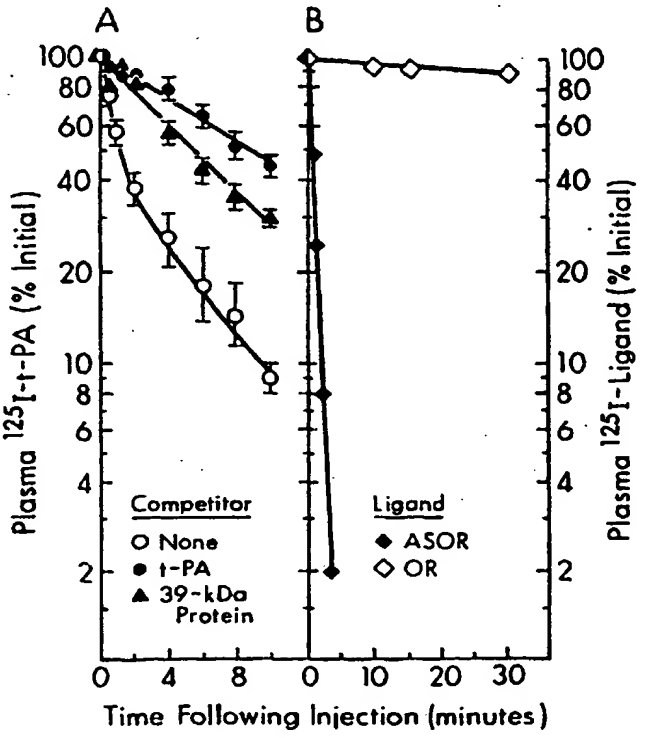
(54) Title: METHODS AND COMPOSITIONS FOR INHIBITION OF HEPATIC CLEARANCE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR

(57) Abstract

Methods and compositions for inhibiting the hepatic clearance of tissue-type plasminogen activator (t-PA) *in vivo* by administering a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof, or genetically or chemically modified forms of the 39kDa protein or fragments thereof are described. Compositions for treatment of thrombolytic diseases comprised of t-PA and a t-PA-hepatic clearance-inhibiting effective amount of 39kDa protein, a t-PA-hepatic clearance inhibiting fragment thereof, and genetically or chemically modified forms of the 39kDa protein or its fragments are described.



TITLE

METHODS AND COMPOSITIONS FOR INHIBITION OF HEPATIC CLEARANCE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR

TECHNICAL BACKGROUND

This invention was made with U.S. Government support under HL 17646 awarded by the National Institutes of Health. The U.S. Government has certain rights in the invention.

FIELD OF THE INVENTION

This invention relates to methods and compositions for inhibiting the hepatic clearance of tissue-type plasminogen activator (t-PA) *in vivo* comprising administering a t-PA-hepatic clearance-inhibiting amount of 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof, or genetically or chemically modified forms of the 39kDa protein or fragments thereof; and to methods and compositions for treatment of thrombolytic diseases comprising administering t-PA and a t-PA-hepatic clearance-inhibiting effective amount of 39kDa protein, a t-PA-hepatic clearance inhibiting fragment thereof, and genetically or chemically modified forms of the 39kDa protein or its fragments.

BACKGROUND OF THE INVENTION

Tissue plasminogen activator is an enzyme widely used as a thrombolytic agent in the treatment of acute myocardial infarction. t-PA is secreted from endothelial cells as a single polypeptide chain that is subsequently cleaved (between Arg_{275} and Ile_{276}) into two chains held together by a single disulfide bond (Rijken, D.C. et al., (1981) J. Biol. Chem. 256, 7035-7041. Both the single- and two-chain forms of the enzyme can bind to fibrin (Rijken, D.C. et al., (1982) J. Biol. Chem. 257, 2920-

(1985) Blood 65, 539-544); rats (Emeis, J.J. et al., (1985) Thromb. Haemost. 54, 661-664; Rijken, D.C. et al., (1986) Biochem. J. 238, 643-646; Kuiper, J. et al., (1988) J. Biol. Chem. 263, 18220-18224; Bakhit, C. et al., (1988) Fibrin. 2, 31-36; Krause, J. et al., (1990) Biochem. J. 267, 647-652); rabbits (Korninger, C. et al., (1981) Thromb. Haemostasis 46, 658-661; Bounameaux, H. et al., (1986) Blood 67, 1493-1497); dogs (Devries, S.R. et al., (1987) Fibrin. 1, 17-21; Yasuda, T. et al., (1988) J. Clin. Invest. 81, 1284-1291); and monkeys (Flameng, W. et al., (1985) J. Clin. Invest. 75, 84-90). These studies together with experiments in man (Garabedian, H.D. et al., (1986) Am. J. Cardiol. 58, 673-679; Verstraete, M. et al., (1986) Thromb. Haemostas. 56, 1-15) demonstrate the rapid removal of t-PA from the circulation, which varies from about $t_{1/2}$ = 1 min in rats to $t_{1/2}$ = 5 min in man.

The liver appears to be the major site of removal and catabolism of t-PA (Nilsson, T. et al., (1984) Scand. J. Haematol. 33, 49-53; Devries, S.R. et al., (1987) Fibrin. 1, 17-21; Korninger, C. et al., (1981) Thromb. Haemostasis 46, 658-661; Bounameaux, H. et al., (1986) Blood 67, 1493-1497; Beebe, D.P. et al., (1986) Thromb. Res. 43, 663-674; Nilsson, S. et al., (1985) Thromb. Res. 39, 511-521; Emeis, J.J. et al., (1985) Thromb. Haemost. 54, 661-664; Rijken, D.C. et al., (1986) Biochem. J. 238, 643-646; Fuchs, H.E. et al., (1985) Blood 65, 539-544; and Kuiper, J. et al., (1988) J. Biol. Chem. 263, 18220-18224). About 80% of exogenous t-PA delivered intravascularly rapidly accumulates in the liver and is subsequently degraded, with subsequent appearance of degradation

uptake of labelled t-PA in isolated liver endothelial cells. The endocytosis of t-PA is mediated, at least in part, by mannose receptors on endothelial cells (Einarsson, M. et al., (1985) Thromb. Haemost. 54, 270; and Kuiper, J. et al., (1988) Fibrin. 2, 28). Monensin, NH_4Cl, and cytochalasin B block the uptake and degradation of t-PA, indicating that the uptake is endocytotic and that the degradation is lysosomal. In hepatoma cell lines, representing parenchymal cells, t-PA clearance involves ligand binding, uptake, and degradation mediated by a high capacity, high-affinity specific receptor system (Owensby, D.A. et al., (1988) J. Biol. Chem. 263, 10587-10594). Subfractionation of rat liver parenchymal, endothelial, and Kupffer cells 5 minutes after ^{125}I-t-PA injection revealed that liver parenchymal cells are responsible for about 55% of the cleared ^{125}I-t-PA, endothelial cells for about 40%, and Kupffer cells for about 5% (Kuiper, J. et al., (1988) J. Biol. Chem. 263, 18220-18224; Rijken, D.C. et al., (1990) Thromb. Res. Suppl. X, 63-71).

Two distinct mechanisms for t-PA catabolism by hepatoma cells have been shown. t-PA complexed to PAI-1 is recognized by a PAI-1 dependent receptor on the cell surface of human hepatoma HepG2 cells (Schwartz, A.L. et al., (1981) J. Biol. Chem. 256, 8878-8881; Owensby, D.A. et al., (1988) J. Biol. Chem. 263, 10587-10594; Morton, P.A. et al., (1989) J. Biol. Chem. 264, 7228-7235; Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602). t-PA in the absence of bioactive PAI-1 has been found to bind to PAI-1 independent receptors which mediate binding and endocytosis of t-PA on rat hepatoma MH_1C_1 cells (Bu,

thrombolytic diseases which allows t-PA to be administered in much smaller doses than currently required. These and other objectives and features of the invention will be apparent from the following description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a graph of the saturation binding of ^{125}I-39kDa protein to MH_1C_1 cells in the presence or absence of 0.5μM unlabeLled 39kDa protein. (*circles*) = total ligand binding; (*triangles*) = non-specific ligand binding. Specific binding (*squares*) was calculated as the difference between total and non-specific binding. Each symbol represents the mean of triplicate determinations. The *inset* is a Scatchard plot of specific binding where B = bound ^{125}I-39kDa protein and B/F = bound/free ^{125}I-39kDa protein. FIG. 1B is a SDS-PAGE gel (10% acrylamide, non-reduced) of equivalent volumes of post-binding cell lysates, each from about 60,000 cells. The position of ^{125}I-39kDa protein is indicated by a closed arrow.

FIGS. 2A-B are graphs of the saturation binding of ^{125}I-t-PA and ^{125}I-39kDa protein to HepG2 cells. FIG. 2A is a graph of the saturation binding of ^{125}I-t-PA in the absence or presence of 1 μM unlabelled t-PA. (*squares*) = total ligand binding; (*triangles*) = non-specific ligand binding; (*circles*) = specific binding calculated as the difference between total and non-specific binding. *Symbols* represent the means of triplicate determinations. The *inset* is a Scatchard plot of specific binding, where B = bound ^{125}I-t-PA, B/F = bound/free ^{125}I-t-PA. FIG. 2B is a graph of the saturation binding of the ^{125}I-39kDa

FIG. 5A is a graph of the inhibition of 3 nM ^{125}I-t-PA binding to HepG2 cells by the 39kDa protein in the absence or presence of increasing concentrations of competitor proteins. Each symbol represents the average of duplicate determinations and the standard deviations are less than 5%. FIG. 5B is a SDS polyacrylamide gel (7.5%) of post-binding buffers overlying cell monolayers and corresponding cell lysates where the gel was analyzed under nonreducing conditions using a sample buffer containing low concentration of SDS (0.2%). Samples were selected from either no competitor protein (lanes 1 and 5), or with 500 nM t-PA (lanes 2 and 6), 500 nM 39kDa protein (lanes 3 and 7), or 500 nM BSA (lanes 4 and 8).

FIG. 6 are SDS-PAGE gels of chemical cross-linking of t-PA:PAI-1 to LRP on ^{35}S-methionine-labelled HepG2 cells which were analyzed under nonreducing or reducing conditions. (N.R.) = normal rabbit serum (*lanes 1, 6, 11*); (α-t-PA) = anti-t-PA antibody (*lanes 2, 7, 12*); (α-PAI-1) = anti-PAI-1 antibody (*lanes 3, 8, 13*); (α-39kDa) = anti-39kDa protein antibody (*lanes 4, 9, 14*); (α-LRP) = anti-LRP antibody (*lanes 5, 10, 15*). The regions of cross-linked material in the non-reducing gel are marked with brackets. The following positions are indicated: LRP large subunit (515 kDa) (*2 closed arrowheads*); LRP small subunit (85 kDa) (*1 closed arrowhead*); PAI-1 (*1 closed arrow*); and t-PA:PAI-1 complex (*1 open arrow*).

FIGS. 7A-B are SDS-PAGE gels (8.5% acrylamide) of cross-linking of unlabelled 39kDa protein to the cell surface of metabolically labelled rat MH_1C_1 cells. Cell monolayers in lanes 4-6 were incubated for 1 hour with 0.5 mM DTSSP, whereas cell

FIGS. 10A-B are graphs of the inhibition of ^{125}I-t-PA uptake and degradation by the 39kDa protein on HepG2 cells. (*open squares*) = prewarmed (37°C) binding buffer containing ^{125}I-t-PA (3 nM); (*open circles*) = prewarmed (37°C) binding buffer containing ^{125}I-t-PA (3 nM) in the presence of 1 μM concentration of t-PA; (*open triangles*) = prewarmed (37°C) binding buffer containing ^{125}I-t-PA (3 nM) in the presence of 1 μM concentration of the 39-kDa protein; (*closed circles*) = prewarmed (37°C) binding buffer containing ^{125}I-t-PA (3 nM) in the presence of 1 μM concentration of BSA. FIG. 10A shows TCA soluble radioactivity representing the degraded ligands in fmoles equivalents of t-PA. FIG. 10B shows the radioactivity associated with each cell lysate in fmoles equivalents of t-PA. In each graph, each symbol represents the average of triplicate determinations and the standard deviations were less than 5%.

FIGS. 11A-B are graphs of the inhibition of ^{125}I-t-PA binding by rat 39kDa protein on MH_1C_1 cells in the presence of increasing concentrations of either unlabelled t-PA or 39kDa protein. Each point represents the mean of triplicate determinations. FIG. 13A shows the inhibition of ^{125}I-t-PA binding by the 39kDa protein. FIG. 13B shows the inhibition of ^{125}I-t-PA binding by unlabelled t-PA.

FIG. 12 is a graph of the rapid uptake and degradation of ^{125}I-39kDa protein by rat MH_1C_1 cells at 37°C. *Closed circles* represent TCA soluble counts (internalized and degraded ligand) in the extracellular media. *Triangles* indicate the amount of cell associated radioactivity as determined following lysis of the cell monolayers. *Open circles* represent the sum of both

protein. (O) = administration of ^{125}I-39kDa protein alone (n=6); (●) = administration of 12.5 nmol unlabelled 39kDa protein prior to ^{125}I-39kDa protein (n=4); (■) = administration of 50 nmol unlabelled 39kDa protein prior to ^{125}I-39kDa protein (n=4); (▲) = administration of 125 nmol unlabelled 39kDa protein prior to ^{125}I-39kDa protein (n=2).

FIG. 18 is a graph of the inhibition of ^{125}I-t-PA binding to rat liver MH_1C_1 cells by intact 39kDa protein, 20kDa N-terminal fragment and 28kDa C-terminal fragment. (●) = intact 39kDa protein; (■) = the 20kDa N-terminal fragment; (▲) = the 28kDa C-terminal fragment; and (O) = PVDF-elution buffer alone.

SUMMARY OF THE INVENTION

The present invention provides a method of inhibiting the hepatic clearance of t-PA *in vivo* in humans by the administration of a t-PA-hepatic clearance-inhibiting amount of a 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment thereof. The 39kDa protein and fragments thereof may be genetically or chemically modified.

An advantage of this method is that the plasma half-life of t-PA is significantly increased.

The present invention also provides a 28kDa protein and a chemically synthesized gene encoding this protein. It has been found that this 28kDa protein inhibits hepatic clearance of t-PA.

The present invention also provides a method of thrombolysis in a mammal by the administration of a thrombolytically effective amount of t-PA and a t-PA-hepatic

In accordance with the invention, a 39kDa protein which competitively binds the hepatic receptor for t-PA has been found. By binding to the hepatic receptor for t-PA, the 39kDa protein prevents t-PA from being bound to the receptor and removed from the circulating plasma by endocytosis. This increases the plasma half-life of t-PA thereby prolonging t-PA's therapeutic effectiveness. An increase in the plasma half life of t-PA means that a smaller amount of t-PA may be used, which reduces the risk of systemic fibrinolysis and hemorrhage. As t-PA is very expensive, a significant cost savings can be achieved which, in turn, increases the availability of t-PA for clinical use.

The 39kDa protein is an active, effective, competitive binding agent for the hepatic receptor for t-PA. This t-PA-hepatic clearance-inhibiting protein is characterized by binding to LRP and inhibiting the binding of t-PA to LRP up to about 80% (K_i of about 0.5 nM) (see Examples 5-8 and 10, *infra*). Fragments of this 39kDa protein, particularly a 28kDa protein fragment, also competitively bind to the t-PA hepatic receptor. When the 39kDa protein or t-PA-hepatic clearance inhibiting fragment thereof is employed in the present invention, the standard dose of t-PA is reduced up to 90%.

Hepatic clearance of t-PA is inhibited *in vivo* in humans by administering a t-PA-hepatic clearance-inhibiting amount of the 39kDa protein or a t-PA-hepatic clearance-inhibiting fragment of the 39kDa protein. The mode of administration is preferably intravenous. The preferred amount of 39kDa protein or fragment administered to the human to

```
                  140                 150                 160
E E L D K L W R E F L H H K E K V H E Y N V L L E T L S R T E E I

          170                 180                 190
H E N V I S P S D L S D I K G S V L H S R H T E L K E K L R S I N

  200                 210                 220                 230
Q G L D R L R R V S H Q G Y S T E A E F E E P R V I D L W D L A Q

                  240                 250                 260
S A N L T D K E L E A F R E E L K H F E A K I E K H N H Y Q K Q L

          270                 280                 290
E I A H E K L R H A E S V G D G E R V S R S R E K H A L L E G R T

    300                 310                 320
K E L G Y T V K K H L Q D L S G R I S R A R H N E L
```

The 39kDa protein was prepared as shown in Example 1.

EXAMPLE 1

Purification of the 39kDa Protein

The procedure for purification of the 39kDa protein from strains of *E.coli* carrying the over-expression plasmid *pGEX-39kDa* has been described in Herz, J. et al., (1991) J. Biol. Chem. 266, 21232-21238. A modified version of that procedure, described below, was employed.

Cultures of *E.coli* strain DH5α carrying the over-expression plasmid *pGEX-39kDa* were grown to mid-log phase in LB medium with 100 μg/ml ampicillin at 37°C. Cultures were cooled to 30°C and supplemented with 0.01% isopropylthio-β-D-galactoside to induce expression of the glutathione-S-transferase-39kDa fusion protein. Following a 4-6 hour induction at 30°C, cultures were cooled on ice and collected by centrifugation.

All of the following steps were carried out at 4°C. Cell pellets were lysed in PBSa with 1% Triton X-100, 1 μM pepstatin, 2.5 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl

200 209
L S G R I S R A R H N E L

The 28kDa protein is characterized by a molecular weight of 28,000 daltons on SDS-PAGE, stability to acid hydrolysis, solubility in 1% Triton X-100, and having approximately the same inhibitory activity (K_i) on t-PA binding to the hepatic receptor as the 39kDa protein. The 28kDa protein may be cloned and purified as shown in the following example.

EXAMPLE 2

Cloning of the 28kDa Protein

The 28kDa protein is produced with a bacterial expression system. The gene encoding this protein is synthesized using polymerase chain reaction (PCR) with the following primers set forth in the Sequence Listing as SEQ ID NO: 3 and SEQ ID NO: 4, respectively:

5'CCGCGTGGATCCCCCAGGCTGGAAAAGCTGTGG3',

5'TCAATGAATTCTCAGAGTCGCTCGCCGTCGCCCAC3'.

These PCR primers contain built-in restriction sites (BamH1 and EcoR1, respectively). The PCR product after restriction enzyme digestion is cloned directly to the pGEX-2T vector (Pharmacia). Other bacterial expression vectors may be used. The constructed plasmid is used to transform bacteria *E. coli* strain DH5αF' and this bacterial transformant bearing the recombinant plasmid is used to produce the 28kDa protein using the procedure of Example 1.

* * * * *

Using standard recombinant techniques, a chemically synthesized gene encoding the 28kDa protein may be prepared. The chemically synthesized gene comprises a chemically

inhibiting fragment thereof. The preferred amount of t-PA is between 0.15 and 1.5 mg/kg of body weight/dose. The preferred amount of 39kDa protein or fragment administered to the mammal is between 60 to 6,000 mg/kg of body weight/dose. When the fragment of the 39kDa protein is the 28kDa protein, the preferred amount administered to the mammal is between 38 to 3,800 mg/kg of body weight/dose. The 39kDa protein or fragment thereof may be administered to the mammal up to 20 minutes prior to administering the t-PA.

A composition for treating thrombolytic diseases which may be employed in the method of thrombolysis includes an effective amount of t-PA and an effective amount of a t-PA hepatic clearance-inhibiting 39kDa protein or a t-PA hepatic clearance-inhibiting fragment thereof. The t-PA is preferably present in a dosage amount of from about 0.15 to 1.5 mg/kg of body weight/dose. The 39kDa protein or fragment thereof is present in a dosage amount of from about 60 to 6,000 mg/kg of body weight/dose. When the fragment of the 39kDa protein is the 28kDa protein, the dosage amount is from about 38 to 3,800 mg/kg of body weight/dose.

t-PA is primarily used in the treatment of thrombolytic diseases, but t-PA has also been used for treating myocardial infarctions, tumors via fibrinolysis, and sickle cell anemia via fibrinolysis. The present invention accordingly provides a pharmaceutical composition for a mammalian patient containing t-PA, the improvement comprising further including a t-PA hepatic clearance-inhibiting amount of 39kDa protein or a t-PA hepatic clearance-inhibiting fragment thereof. The t-PA is

Products) using the Iodogen procedure described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602. Specific radioactivities for each radiolabelled protein were between 1 and 2 x 10^7 cpm/μg of protein as measured by γ scintillation spectrometry.

Saturation Binding Analysis

Cells were grown in 12 well dishes to approximately 10^6 cells per well. Monolayers were taken directly from 37°C and cooled on ice. The binding buffer used for the recombinant 39kDa protein was PBSc (phosphate-buffered saline supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$). Each cell monolayer was washed three times with PBSc prior to the addition of binding buffer containing various concentrations of radiolabelled ^{125}I-39kDa protein, either in the presence or absence of an excess of unlabelled protein. Cell monolayers were incubated at 4°C with between 1 and 24 nM recombinant ^{125}I-39kDa protein, either in the presence or absence of 0.5 μM unlabelled protein.

Following a 90 minute incubation, cell monolayers were washed three additional times with PBSc to remove non-specifically associated ligand, and lysed in "low SDS lysis buffer" (0.0625 M Tris-HCl, pH 6.8, with 0.2% sodium dodecyl sulfate, 10% glycerol, and 0.01% bromophenol blue), as described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602. The amount of cell associated ligand was determined by γ scintillation spectrometry. In cases where t-PA and the 39kDa protein were co-bound, the t-PA binding buffer (PBSa containing 0.2 mM $CaCl_2$ and 10 mM ϵ-amino-n-caproic acid) was used as

Table 1

Specific Binding of ^{125}I-39kDa Protein and ^{125}I-t-PA to MH_1C_1 Cells

ligand	preincubation	Bmax ± S.D. (10^3 sites/cell)	Kd ± S.D. (nM)	Experiment (n)
A. Summary				
^{125}I-39kDa	none	380 ± 190	3.3 ± 0.9	10
^{125}I-t-PA	none	78 ± 35	4.9 ± 1.3	7
B. Simultaneous Assays				
^{125}I-39kDa	none	390 ± 150	3.5 ± 0.9	4
^{125}I-t-PA	none	60 ± 35	5.6 ± 1.5	4
C. Simultaneous Assays Performed in the Presence or Absence of Saponin				
^{125}I-39kDa	none	250 ± 8	2.7 ± 1.3	3
^{125}I-t-PA	saponin	515 ± 24	9.1 ± 3.6	3

These data indicate both specific and saturable binding of the 39kDa protein to a single kinetic species of high affinity receptor on the MH_1C_1 cell surface.

The nature of the 39kDa protein binding species was shown by visualizing cell lysates from each binding experiment by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (FIG. 1B). Under the mild conditions used in these experiments (0.2% SDS), the 39kDa protein appeared to bind in isolation, without apparent association with any auxiliary binding proteins. These experiments demonstrate that there is a specific and saturable interaction between the 39kDa protein and an MH_1C_1 cell surface molecule(s).

12 hours prior to use. Cell monolayers were generally cultured for two days before use at 80-90% confluence.

Production and Isolation of 39kDa Protein

The 39kDa protein was produced and isolated in accordance with the procedure of Example 1.

Protein Iodination

Iodination of t-PA and 39kDa protein were performed using IODOGEN (Pierce Chemical Co.) as described in Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602. The specific activity was generally 5-10 μCi/μg of protein as determined by γ scintillation spectrometry. The unincorporated ^{125}I after gel-filtration purification over a PD-10 column (Pharmacia) was less than 2% of the total radioactivity.

Saturation Binding Assays

Cells were seeded into multiwell (12 wells/plate) disposable plastic tissue culture plates 2 days prior to assay. Ligand binding buffer for t-PA was composed of phosphate-buffered saline (PBS) supplemented with 0.2 mM $CaCl_2$ and 10 mM EACA, whereas PBSc (PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$) was used for 39kDa protein binding. Binding experiments were performed at 4°C to prevent possible concomitant internalization during the binding interval. Cell monolayers were washed three times on ice with prechilled binding buffer. Binding was initiated by adding 0.5 ml binding buffer containing selected concentrations of ^{125}I-labelled ligand in the absence or presence of an excess unlabelled ligand (1 μM). After incubation at 4°C for 1.5 hours, buffer containing unbound ligand was removed. Cells

in the form of the ^{125}I-t-PA:PAI-1 complex. This is consistent with PAI-1-dependent ^{125}I-t-PA binding on HepG2 cells. (See, Morton, P. A. et al., (1989) J. Biol. Chem. 264, 7228-7235; Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602).

Specific binding of ^{125}I-39kDa protein to HepG2 cells was performed over the concentration range of 1-24 nM. As shown in FIG. 2B, the 39kDa protein specifically bound to HepG2 cells with a low level of non-specific binding. Saturation of specific binding was observed at ^{125}I-39kDa protein concentrations in excess of 12 nM. Scatchard analysis of the binding data from five such experiments yielded 197,000 ± 23,000 (S.D.) homogeneous high affinity surface binding sites per cell with an apparent K_d = 5.1 ± 0.8 nM (S.D.) (166,000 binding sites per cell and K_d = 4.9 nM for the experiment shown in FIG. 2B). When post-binding cell lysates were analyzed by SDS-PAGE, ^{125}I-39kDa protein was observed at its native molecular mass (i.e., 39kDa) indicating that the binding did not include an SDS-stable complex with other proteins.

* * * * *

The following example demonstrates that low density lipoprotein receptor related protein (LRP) is a receptor for the 39kDa protein.

EXAMPLE 5

Cross-linking of ^{125}I-39kDa Protein to the MH_1C_1 Cell Surface

The 39kDa protein was originally isolated via its ability to co-purify with a low density lipoprotein receptor related protein (LRP) by α2-macroglobulin affinity

Labelling was carried out for 4-5 hours at 37°C. After metabolic labelling, cell monolayers were cooled on ice, washed three times with cold PBSc, and incubated with 14 nM of the radiolabelled ^{125}I-recombinant 39kDa protein of Example 3, either in the presence or absence of an excess of unlabelled 39kDa protein, to allow specific binding to the cell surface receptor.

Chemical Cross-Linking

Saturation binding was performed as described above, but with 10 cm dishes of MH_1C_1 cells. Either unlabelled or ^{35}S-methionine labelled cells were used. Following incubation with radiolabelled and unlabelled ligands (39kDa proteins), cell monolayers were washed three times with PBSc to remove non-specifically associated ligand and incubated with either PBSc alone or PBSc containing 0.5-1 mM dithio*bis*(sulfosuccinnimidylpropionate) (DTSSP) (Pierce Chemical Co.), a disulfide bond containing, thiocleavable cross-linker. After one hour, cross-linking reactions were quenched by three washes with tris-buffered saline, and cell monolayers were solubilized in PBSa containing 1% Triton X-100 and 1 mM PMSF. Immunoprecipitations were performed as described below.

Immunoprecipitation

Aliquots of cell lysates from cross-linking and metabolic labelling experiments were added to equal volumes of PBSc containing 1% Triton X-100, 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate (SDS), 0.5% bovine serum albumin , and 1mM PMSF (immunomix). When metabolically labelled cells were

(arrowhead) was immunoprecipitated only with α-39kDa serum. Samples electrophoresed under either reducing or non-reducing conditions show that the α-LRP antibody was unable to immunoprecipitate the radiolabelled 39kDa ligand in the absence of cross-linker. Since there was a marked decrease in the amount of ^{125}I-39kDa protein bound in the presence of an excess of unlabelled protein, the association of ligand with its cell surface receptor was shown to be specific. In FIG. 3A and 3B, the radiolabelled 39kDa protein was both bound and cross-linked to the MH_1C_1 cell surface. When samples from each immunoprecipitation were analyzed under non-reducing conditions (FIG. 3A), the complex of ligand associated radioactivity was immunoprecipitable with both α-LRP and α-39kDa antibodies. This complex was of very high apparent molecular weight, remaining largely in the stacking gel during electrophoresis (arrows). Again, this interaction was shown to be specific, as the association of ^{125}I-39kDa with this high molecular weight complex was abrogated in the presence of an excess of unlabelled 39kDa protein. When these same samples were electrophoresed in the presence of 5% 2-mercaptoethanol to dissociate the cross-linker (FIG. 3B), a single 39kDa binding species was seen.

The foregoing experiments demonstrate that there is a specific interaction between the 39kDa protein and the MH_1C_1 cell surface which is mediated, at least in part, by an association with LRP.

this example, protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard. Cell number was determined by counting cells with a hemocytometer.

Metabolic Labelling

Cells growing in 10 cm dishes at about 80% confluence were incubated for 30 min at 37°C in two changes of Earle's minimum essential medium lacking L-methionine and containing 2 mM L-glutamine (Gibco Laboratories). Metabolic labelling was initiated by the addition of the above medium supplemented with ^{35}S-methionine (400 μCi/ml)(Amersham). Following incubation for 5 hours at 37°C, cell monolayers were washed with binding buffer and were used for ligand binding and chemical cross-linking experiments as described below.

Chemical Cross-linking

Experiments were performed with either ^{125}I-labelled ligand cross-linked to unlabelled cells or unlabelled ligand cross-linked to ^{35}S-methionine metabolically-labelled cells. After ligand binding at 4°C, each cell monolayer was washed three times with PBSc. Chemical cross-linking was performed by incubating the cell monolayer with PBSc containing 0.5 mM dithio*bis*(sulfosuccinnimidylpropionate) (DTSSP) (Pierce Chemical Co.). After 30 min at 4°C, the reaction was quenched by washing the cell monolayer two times with Tris-buffered saline (TBS). Cells were then solubilized in PBSc containing 1% (v/v) Triton X-100 (Sigma Chemical Co.) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co.) ("lysis buffer") for 30 min at 4°C with a brief sonication at

three times with immunomix and three times with PBSc. The protein A-agarose beads were divided into two equal parts before the final wash for nonreducing and reducing assays. The immunoprecipitated material was then released from the beads by boiling each sample for 5 min in 0.0625 M Tris-HCl, pH 6.8, 2% (w/v) SDS, and 10% (v/v) glycerol ("Laemmli sample buffer") (Laemmli, U. K., (1970) Nature 227, 680-685) with or without 5% (v/v) 2-mercaptoethanol, and was analyzed by SDS-PAGE as described below.

SDS-PAGE and Autoradiography

Samples of cell lysates or immunoprecipitations were analyzed by SDS-PAGE using polyacrylamide slab gels as described in Laemmli, U. K., (1970) Nature 227, 680-685, under reducing or non-reducing conditions. The following pre-stained molecular weight standards from Bio-Rad were used: myosin: 205 kDa; β-galactosidase: 117 kDa; bovine serum albumin: 80 kDa; ovalbumin: 50 kDa. Autoradiography of ^{125}I-labelled proteins was performed with dried polyacrylamide gels using Hyperfilm-MP (Amersham). For fluorography of ^{35}S-labelled proteins, gels were impregnated with Amplify (Amersham), dried, and exposed to films. Films were placed at -70°C for various periods of time as specified in each figure prior to developing.

FIG. 4 shows an experiment with ^{125}I-t-PA binding and cross-linking to HepG2 cells. Cell lysates without or with cross-linking were immunoprecipitated with one of the following antibodies: normal rabbit serum, anti-t-PA antibody, anti-PAI-1 antibody, or anti-LRP antibody. The

receptor complexes consisting of ^{125}I-t-PA:PAI-1 and LRP appeared as a broad smear migrating on top of a 7% SDS-polyacrylamide gel with a molecular mass estimated to be greater than 600 kDa. However, when the same material was analyzed by SDS-PAGE under reducing conditions, the radioligand ^{125}I-t-PA:PAI-1 complex and ^{125}I-t-PA appeared following reduction of the thiocleavable cross-linker DTSSP (FIG. 4B). Reduction of the cross-linked material caused additional dissociation of the ^{125}I-t-PA:PAI-1 complex resulting in an even greater amount of free ^{125}I-t-PA. Normal rabbit serum showed no specific interaction with any of the cross-linked material (lanes 1 and 5).

To exclude the possibility that chemical cross-linking of ^{125}I-t-PA:PAI-1 to HepG2 cells generated an antigenic epitope for anti-LRP antibody, unlabelled t-PA was cross-linked to ^{35}S-methionine metabolically-labelled HepG2 cells. This approach allowed direct visualization of the binding protein for the t-PA:PAI-1 complex or the 39kDa protein and the determination of its relationship to LRP. Following ^{35}S-methionine metabolic labeling of HepG2 cells, cell monolayers were incubated with binding buffer alone, or binding buffer containing t-PA (15 nM) or the 39kDa protein (15 nM) (FIG. 6). Cell monolayers in the absence of ligand was lysed directly without chemical cross-linking whereas those following ligand binding were subjected to chemical cross-linking with DTSSP. Each of the cell lysate preparations was then immunoprecipitated with one of the following antibodies: normal rabbit serum (lanes 1, 6, 11),

When the ^{35}S-methionine-labelled HepG2 cell monolayer was exposed to externally added 39kDa protein, the ^{35}S-methionine-labelled ligand-receptor complex appearing on top of the gel was immunoprecipitable not only by anti-LRP antibody (lanes 15), but also by anti-39kDa protein antibody (lanes 14). Analysis of these cross-linked materials under reducing conditions yielded the 515kDa radiolabelled LRP band when immunoprecipitated with anti-39kDa antibody, further demonstrating the receptor protein for 39kDa protein on HepG2 cells is LRP.

EXAMPLE 7

The 39kDa Protein is Cross-linked Predominately to LRP on the MH_1C_1 Cell Surface

It was found that LRP is the predominant 39kDa protein binding species by cross-linking unlabelled 39kDa protein to the surface of radiolabelled MH_1C_1 cells using the procedures of Example 5 with the following modifications. Labelling was carried out at 37°C for 4-5 hours in methionine deficient Earle's MEM media supplemented to 0.4 mCi/ml with ^{35}S-methionine (Amersham Corp.). Following several washes, labelled monolayers were bound with unlabelled 39kDa protein, and either cross-linked by the addition of 0.5 mM DTSSP, or incubated in PBSc alone. Lysis and immunoprecipitation were carried out as described above in Example 5.

FIG. 7 shows the results from a typical experiment. When radiolabelled MH_1C_1 cells were cross-linked to unlabelled 39kDa protein, a complex of very high apparent molecular weight resulted, which was immunoprecipitable with both the α-39kDa and, to a lesser degree, the α-human LRP affinity

labelled cell lysates with α-human LRP antibodies results in only the 520kDa (*closed arrow*) and the 85kDa (*open arrow*) subunits of the LRP receptor. Again, no high molecular weight complex was evident. Samples electrophoresed under both reducing and non-reducing conditions gave similar results.

This example demonstrates that the LRP is the predominant 39kDa protein binding species on the MH_1C_1 cell surface.

* * * * *

Example 8 demonstrates that both the 39kDa protein and t-PA specifically co-bind to a single LRP molecule.

EXAMPLE 8

t-PA and the 39kDa Protein can be Cross-linked to the Same LRP Receptor Complex

0.5nM ^{125}I-39kDa protein and 20nM unlabelled t-PA were co-bound to MH_1C_1 cells both in the presence and absence of an excess of unlabelled 39kDa protein. Following several washes to remove non-specifically associated ligand, cell monolayers were either incubated in 1mM DTSSP or in PBSc alone. Once cross-linked, cell monolayers were washed, lysed and immunoprecipitated as described above.

FIG. 8 shows the results from a typical experiment. When the ^{125}I-39kDa protein and unlabelled t-PA were co-bound to the MH_1C_1 cell surface and incubated in the absence of cross-linker (FIG. 8C and D), the radiolabelled 39kDa protein was immunoprecipited from cell lysates with only the α-39kDa antibody (*arrowhead*). Neither the α-LRP antibody nor the α-t-PA antibody showed any affinity for the radiolabelled 39kDa ligand. Samples electrophoresed under both reducing and

This experiment shows that the simple cross-linking of radiolabelled 39kDa protein to the MH_1C_1 cell surface does not change its specificity for either the α-t-PA or α-39kDa antibodies. A number of additional co-binding and cross-linking experiments, performed using ^{125}I-t-PA and unlabelled 39kDa protein, gave similar results (data not shown). This data taken together with the data presented above leads to the conclusion that the 39kDa protein and t-PA can bind simultaneously to LRP on the MH_1C_1 cell surface.

* * * * *

It was found from the following examples that endocytosis of the 39kDa protein is receptor-mediated like t-PA and that the 39kDa protein modulates the receptor-mediated endocytosis of t-PA.

EXAMPLE 9

Rapid Uptake and Degradation of ^{125}I-39kDa by MH_1C_1 Cells

It is known that MH_1C_1 cells rapidly endocytose and degrade t-PA (Bu, G. et al., (1992) J. Biol. Chem. 267, 15595-15602). The following single cycle uptake and degradation experiments demonstrate that MH_1C_1 cells similarly endocytose the 39kDa protein.

Endocytosis experiments were performed according to Ciechanover et al., (1983) J. Biol. Chem. 258, 9681-9689 and Owensby et al., (1988) J. Biol. Chem. 263, 10587-10594. 6 well dishes containing 10^6 cells/well were preincubated at 4°C with 14 nM ^{125}I-39kDa protein to allow binding. Following three washes with PBSc (4°C) to remove non-specifically associated ligand, each dish was incubated at 37°C, and a pre-warmed

appeared in the overlying media initially at 10 minutes, with plateau levels reached at between 45 and 60 minutes. This kinetic pattern of uptake and degradation of a single cohort of pre-bound ^{125}I-39kDa ligand was identical to that observed for ^{125}I-t-PA with these MH_1C_1 cells.

EXAMPLE 10

Inhibition of ^{125}I-t-PA Uptake and Degradation by the 39kDa Protein on HepG2 Cells

The receptor-mediated endocytosis of the t-PA:PAI-1 complex by HepG2 cells has been previously demonstrated. (See, Owensby, D. A. et al., (1988) J. Biol. Chem. 263, 10587-10594; Morton, P. A. et al., (1989) J. Biol. Chem. 264, 7228-7235; Underhill, D. M. et al., (1992) Blood 80, 2746-2754). It was found from the following experiment that the endocytosis and degradation of the t-PA:PAI-1 complex by HepG2 cells were also via LRP and that the 39kDa protein inhibits the endocytosis and degradation of ^{125}I-t-PA.

Throughout this example, protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard. Cell number was determined by counting cells with a hemocytometer.

Ligand Uptake and Degradation

HepG2 cell monolayers seeded in 6-well dishes were incubated at 37°C with 3 nM ^{125}I-t-PA in the absence or presence of various competitors at selected concentrations. After selected intervals, binding buffer overlying cell monolayers was collected, cell monolayers were chilled on ice and washed with prechilled binding buffer for three times to prevent further ligand uptake and degradation. Cellular degradation

associated radioactivity was quantitated during the first hour of endocytosis. As shown in FIG. 10B, about 70% of the specific ^{125}I-t-PA uptake was inhibited by the 39kDa protein, whereas the control protein BSA showed no inhibitory effect on this process.

These results clearly demonstrate that LRP serves as the major PAI-1-dependent endocytosis receptor for t-PA on HepG2 cells, and that the 39kDa LRP receptor-associated protein modulates this interaction.

* * * * *

MH_1C_1 cells rapidly catabolize the 39kDa protein as shown in Example 11.

EXAMPLE 11

Rate of 39kDa Protein Uptake by MH_1C_1 Cells

The following experiment used the procedure described in Schwartz, A.L. et al., (1982) J. Biol. Chem. 257, 4230-4237.

Rate of 39kDa Protein Uptake

Dishes containing monolayers of 10^6 MH_1C_1 cells/well were cooled on ice and washed three times with cold PBSc. Ligand uptake was initiated by the addition of prewarmed (37°C) binding buffer containing 28 nM ^{125}I-39kDa protein, either in the presence or absence of an excess of unlabelled 39kDa protein. Each dish was incubated at 37°C for a period of between 0 and 120 minutes. At each time point, one of the dishes was cooled directly on ice, and the overlying media was removed. Cell monolayers were washed three times with PBSc and lysed in low SDS lysis buffer for the determination of

of either unlabelled t-PA or recombinant rat 39kDa protein as competitors. Unlabelled t-PA inhibits binding of ^{125}I-t-PA with an apparent K_i value of 8 nM (FIG. 12B). The 39kDa protein, on the other hand, competed at a much lower concentration, with an apparent K_i value of 0.5 nM (FIG. 12A). This experiment demonstrates that the 39kDa protein is a strong competitor for ^{125}I-t-PA binding to LRP on the MH_1C_1 cell surface.

EXAMPLE 13

Inhibition of ^{125}I-t-PA:PAI-1 Complex Binding to HepG2 Cells by 39kDa Protein and Anti-LRP Antibody

In the following ligand binding competition experiments, it was found that PAI-1-dependent ^{125}I-t-PA binding was also mediated via LRP. Specific binding of 3 nM ^{125}I-t-PA was performed in the absence or presence of each of three competitor proteins: t-PA, 39kDa protein, or bovine serum albumin (BSA). Each competitor protein was included at increasing concentrations up to more than 100-fold molar excess of the radiolabelled ligand. Throughout this example, protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard. Cell number was determined by counting cells with a hemocytometer.

Ligand binding competition assays

Binding of the ^{125}I-labelled ligand was performed as described above in Example 4 in the absence or presence of various competitors at selected concentrations. In those experiments in which both t-PA and 39kDa protein were involved, the t-PA binding buffer was used. Nonspecific binding was determined in the presence of 1 μM unlabelled ligand.

overlying buffer shown in lane 1-4 were also analyzed via the same SDS polyacylamide gel (FIG. 5B). Binding of the ^{125}I-t-PA:PAI-1 complex to HepG2 cells is demonstrated in the cell lysates without competitor protein (lane 5). When excess unlabelled t-PA was included, however, specific binding of the ^{125}I-t-PA:PAI-1 complex was absent (lane 6). When the cell lysate with 500 nM 39kDa protein was compared to that with ^{125}I-t-PA alone, specific ^{125}I-t-PA:PAI-1 complex binding was reduced by about 80% (lane 7). The control protein BSA neither interfered with the complex formation (lane 4) nor inhibited specific ^{125}I-t-PA:PAI-1 complex binding (lane 8). These results thus demonstrate that the inhibition of ^{125}I-t-PA binding to HepG2 cells by the 39kDa protein occurs at the level of ^{125}I-t-PA:PAI-1 binding to LRP without interference with the initial complex formation.

Modulation of ligand binding to LRP by the 39kDa protein is not due to competition at a single site with various ligands since previous experiments have shown that LRP ligands (e.g., α_2M^*, t-PA) compete only slightly with the 39kDa protein for binding to LRP. (See, Bu, G. et al., (1992) Proc. Natl. Acad. Sci. USA 89, 7427-7431). To test whether ^{125}I-39kDa protein binding is affected by t-PA on HepG2 cells, ligand binding competition experiments were performed. Competitor proteins (t-PA, 39kDa protein, or BSA) at various concentrations were included in ^{125}I-39kDa protein binding. As shown in FIG. 13, specific binding of ^{125}I-39kDa protein was reduced only slightly (about 20%) by excess unlabelled t-PA, whereas the binding was completely inhibited by excess

EXAMPLE 14

Effect of 39kDa Protein on Hepatic Clearance of t-PA

39kDa Protein Preparation

The 39kDa protein was isolated from 5 liters of *E. coli* containing the recombinant plasmid according to Example 1 and yielded the GST-39kDa protein. Following thrombin cleavage and removal of the GST, the 39kDa protein was approximately 95% pure as determined by SDS-PAGE and Coomassie/silver staining. The few percent of non-39kDa protein contained within these preparations were unabsorbed fragments of GST. In order to provide a homogeneously pure preparation of 39kDa protein, this partially purified mixture was applied to a heparin agarose column (Sigma Chemical Co.). Following extensive washing with 100 mM NaCl to remove contaminating proteins, the 39kDa protein was eluted at 0.4 M NaCl. This preparation resulted in the greater than 99% homogenous preparation of 39kDa protein as determined by SDS-PAGE and Coomassie/silver staining and shown in FIG. 15.

General Procedures

The following procedures were used throughout this example.

1. Cell Culture

Rat hepatoma MH_1C_1 cells were cultured in accordance with the procedure of Example 3.

2. Protein Iodination

t-PA, 39kDa protein, GST, α1-acid glycoprotein (orosomucoid, OR), and the asialo form of α1-acid glycoprotein (ASOR) were iodinated with ^{125}I as described in Example 4. The

and 10 minutes. After the 10 minute peripheral sample was collected, a central blood sample was collected by an open cardiac puncture and the visceral organs (liver, kidney, spleen) were rapidly removed to ice. Aliquots of the liver and entire kidney and spleen were counted for radioactivity determination. Heparinized blood samples were centrifuged to separate plasma from blood cells and an aliquot of plasma, generally 25 µl, was spotted onto Whatman 3M paper, dried, precipitated in 10% trichloracetic acid and radioactivity determined in a Packard Gamma Spectrophotometer.

1. Clearance of t-PA

To demonstrate that t-PA is rapidly cleared from the blood plasma following intravenous administration, adult 200 g rats were anesthetized and administered 30 pmol ^{125}I-t-PA via venous injection as described above. Arterial blood samples were collected at the indicated times, separated and plasma radioactivity determined.

As seen in FIG. 16A, following intravenous administration to each of the eight rats of 30 pmol of ^{125}I-t-PA, the plasma ^{125}I-t-PA was rapidly cleared with a $t_{½}$ of approximately one minute. Less than 10% of the ^{125}I-t-PA remained in blood plasma at 10 minutes. Analysis of the liver at 10 minutes following the administration revealed approximately 90% of the initial plasma radioactivity was found in liver.

2. Effect of Excess t-PA on t-PA Clearance

Intravenous administration to three rats of 400-fold molar excess unlabelled t-PA (12 nmol) one minute prior to

The following table, Table 2, shows that the analysis of the liver, kidney and spleen at 10 minutes revealed approximately 70% of the initial radioactivity in liver, 7% in kidney and less than 2% in spleen. Following plasma clearance of ^{125}I-39kDa protein in the rat *in vivo* as described in FIG. 17, the liver, kidney and spleen were harvested at 10 minutes and the radioactivity determined. Each figure is the mean ± S.E.M.

TABLE 2

ORGAN DISTRIBUTION OF ^{125}I-39kDa PROTEIN

Preadministration		^{125}I-39kDa Protein	Liver	Kidney	Spleen
(dose)		(dose)	(% initial radioactivity)		
a.	none	30 pmol	69 ± 5	7 ± 1	2 ± 0.2
b.	12.5 nmol	30 pmol	72 ± 3	12 ± 1	2 ± 0.2
c.	50 nmol	30 pmol	25 ± 6	13 ± 2	2 ± 0.6
d.	125 nmol	30 pmol	23 ± 5	31 ± 10	2 ± 0.3

The capacity for hepatic clearance of 39kDa protein is large, but can be saturated as seen in FIG. 17 and Table 2. Intravenous administration of increasing doses of unlabelled 39kDa protein results in a decrease of the fraction of the initial dose found in the liver at 10 minutes. Concomitantly there is an increasing fraction of the initial dose found in the kidney, which indicates that maximal hepatic capacity of 39kDa protein clearance can be readily achieved *in vivo*. Therefore multiple dosing schedules and/or continuous infusion of 39kDa protein should markedly reduce hepatic t-PA clearance *in vivo*.

pmol ^{125}I-α1-acid glycoprotein (OR), and (b) 5 pmol ^{125}I-asialo-α1-acid glycoprotein (ASOR). Arterial blood samples were collected at the indicated times, separated, and plasma radioactivity determined as described above. As seen in FIG. 16B, for example, α1-acid glycoprotein was cleared with a half-time of greater than 90 minutes, whereas its asialo-derivative was rapidly cleared with half-time of approximately 0.5 minute (see Schwartz et al., (1984) CRC Crit. Rev. Biochem. 16, 207-233).

EXAMPLE 15

Effect of 39kDa Protein Fragments on Hepatic Clearance of t-PA

The following experiment demonstrates that the entire 39kDa protein molecule is not required for inhibition of t-PA binding to the hepatic t-PA receptor. The 39kDa protein was chemically cleaved and the individual fragments isolated. These were then tested for inhibition of ^{125}I-t-PA binding to receptors on rat hepatocyte cells. Throughout this example, protein concentration was determined using a Bio-Rad protein assay with bovine serum albumin as a standard.

Purified 39kDa protein was prepared in accordance with the procedure of Example 14. In order to generate isolated fragments of the 39kDa protein, acid hydrolysis was performed under strict conditions. The 39kDa protein in 0.4M NaCl was incubated for 80 hours at room temperature in 70% formic acid. These acid proteolytic conditions provided for the complete cleavage of the intact 39kDa protein at the Asp-Pro bond at amino acid residues 114-115 and the generation of

radioactivity bound to the MH_1C_1 cells was determined as described in Example 14.

As shown in FIG. 18, the intact 39kDa protein inhibited ^{125}I-t-PA binding with 50% inhibition at approximately 1 nM and 90% inhibition at approximately 8-10 nM, consistent with previous observations (Bu et al., (1992) Proc. Natl. Acad. Sci. USA 89, 7427-7431; Examples 10, 12, 13, and 14 above). The 20kDa N-terminal fragment was essentially without effect (i.e., approximately 10% inhibition at 16 nM; buffer alone exhibited 20-30% inhibition under these identical conditions). In marked contrast the 28kDa C-terminal fragment was an extremely active inhibitor of ^{125}I-t-PA binding with approximately 50% inhibition at 4 nM and 90% inhibition at 16 nM as shown in FIG. 18.

The nearly equipotent activity of the 28kDa C-terminal fragment compared to intact 39kDa protein strongly suggests that a small fragment of the 39kDa protein, which could include a fragment that overlaps the dividing point between the 20kDa N-terminal fragment and 28kDa C-terminal fragment, or a chemically or genetically related/modified form of the molecule, will be recognized by the t-PA hepatic receptor and may serve as a powerful reagent in inhibiting t-PA clearance *in vivo*.

* * * * *

The above description is meant to be illustrative of the present invention, and not limiting thereof. All explanations of the inventors' theory of the invention are for illustrative purposes only.

(B) REGISTRATION NUMBER: 31,088

(C) REFERENCE/DOCKET NUMBER: 00108/064909

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (212) 692-1800

(B) TELEFAX: (212) 692-1900

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 323 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(3) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 209 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Lys Phe
 1               5                  10                  15
Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His His Lys
            20                  25                  30
Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser Arg Thr
        35                  40                  45
Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser Asp Ile
    50                  55                  60
Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu Lys Leu
65                  70                  75                  80
Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser His Gln
                85                  90                  95
Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile Asp Leu
            100                 105                 110
Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys Glu Leu Glu Ala
        115                 120                 125
Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile Glu Lys His Asn
    130                 135                 140
His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys Leu Arg His Ala
145                 150                 155                 160
Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser Arg Glu Lys His
                165                 170                 175
Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr Thr Val Lys Lys
            180                 185                 190
His Leu Gln Asp Leu Ser Gly Arg Ile Ser Arg Ala Arg His Asn Glu
        195                 200                 205
Leu
```

(6) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 203 amino acid residues

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Ser Val
 1               5                  10                  15
Arg Leu Thr Ser Cys Ala Arg Val Leu His Lys Glu Lys Ile His Glu
            20                  25                  30
Tyr Asn Val Leu Leu Asp Thr Leu Ser Arg Ala Glu Glu Gly Tyr Glu
        35                  40                  45
Asn Leu Leu Ser Pro Ser Asp Met Thr His Ile Lys Ser Asp Thr Leu
    50                  55                  60
Ala Ser Lys His Ser Glu Leu Lys Asp Arg Leu Arg Ser Ile Asn Gln
65                  70                  75                  80
Gly Leu Asp Arg Leu Arg Lys Val Ser His Gln Leu Arg Pro Ala Thr
                85                  90                  95
Glu Phe Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala Gln Ser
            100                 105                 110
Ala Asn Phe Thr Glu Lys Glu Leu Glu Ser Phe Arg Glu Glu Leu Lys
        115                 120                 125
His Phe Glu Ala Lys Ile Glu Lys His Asn His Tyr Gln Lys Gln Leu
    130                 135                 140
Glu Ile Ser His Gln Lys Leu Lys His Val Glu Ser Ile Gly Asp Pro
145                 150                 155                 160
Glu His Ile Ser Arg Asn Lys Glu Lys Tyr Val Leu Leu Glu Glu Lys
                165                 170                 175
Thr Lys Glu Leu Gly Tyr Lys Val Lys Lys His Leu Gln Asp Leu Ser
            180                 185                 190
Ser Arg Val Ser Arg Ala Arg His Asn Glu Leu
        195                 200
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Asp Gly Leu Asp Glu Asp Gly Glu Lys Glu Ala Arg Leu Ile Arg Asn
 65                  70                  75                  80
Leu Asn Val Ile Leu Ala Lys Tyr Gly Leu Asp Gly Lys Lys Asp Ala
                 85                  90                  95
Arg Gln Val Thr Ser Asn Ser Leu Ser Gly Thr Gln Glu Asp Gly Leu
            100                 105                 110
Asp Asp Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly
        115                 120                 125
Lys Phe Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His
    130                 135                 140
His Lys Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser
145                 150                 155                 160
Arg Thr Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser
                165                 170                 175
Asp Ile Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu
            180                 185                 190
Lys Leu Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser
        195                 200                 205
His Gln Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile
    210                 215                 220
Asp Leu Trp Asp Leu Ala Gln Ser Ala Asn Leu Thr Asp Lys Glu Leu
225                 230                 235                 240
Glu Ala Phe Arg Glu Glu Leu Lys His Phe Glu Ala Lys Ile Glu Lys
                245                 250                 255
His Asn His Tyr Gln Lys Gln Leu Glu Ile Ala His Glu Lys Leu Arg
            260                 265                 270
His Ala Glu Ser Val Gly Asp Gly Glu Arg Val Ser Arg Ser Arg Glu
        275                 280                 285
Lys His Ala Leu Leu Glu Gly Arg Thr Lys Glu Leu Gly Tyr Thr Val
    290                 295                 300
Lys Lys His Leu Gln Asp Leu Ser Gly Arg Ile Ser Arg Ala Arg His
305                 310                 315                 320
Asn Glu Leu
```

8. A chemically synthesized gene encoding a 28kDa protein comprising a chemically synthesized polynucleotide which codes on expression for the amino acid sequence as set forth in SEQ ID NO: 2:

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Lys Phe
 1               5                  10                  15
Ser Gly Glu Glu Leu Asp Lys Leu Trp Arg Glu Phe Leu His His Lys
            20                  25                  30
Glu Lys Val His Glu Tyr Asn Val Leu Leu Glu Thr Leu Ser Arg Thr
        35                  40                  45
Glu Glu Ile His Glu Asn Val Ile Ser Pro Ser Asp Leu Ser Asp Ile
    50                  55                  60
Lys Gly Ser Val Leu His Ser Arg His Thr Glu Leu Lys Glu Lys Leu
65                  70                  75                  80
Arg Ser Ile Asn Gln Gly Leu Asp Arg Leu Arg Arg Val Ser His Gln
                85                  90                  95
Gly Tyr Ser Thr Glu Ala Glu Phe Glu Glu Pro Arg Val Ile Asp Leu
            100                 105                 110
```

14. The pharmaceutical composition of claim 12, wherein the 39kDa protein or fragments thereof is present in a dosage amount of from about 60 to 6,000 mg/kg of body weight/dose.

15. The pharmaceutical composition of claim 12, wherein the fragment of the 39kDa protein is a 28kDa protein and is present in a dosage amount of from about 38 to 3,800 mg/kg of body weight/dose.

16. A composition for treating thrombolytic diseases in a mammal comprising an effective amount of tissue-type plasminogen activator (t-PA), and an effective amount of a t-PA hepatic clearance-inhibiting 39kDa protein or a t-PA hepatic clearance-inhibiting fragment thereof.

17. The composition of claim 16, wherein the t-PA is present in a dosage amount of from about 0.15 to 1.5 mg/kg of body weight/dose.

18. The composition of claim 16, wherein the 39kDa protein or fragments thereof is present in a dosage amount of from about 60 to 6,000 mg/kg of body weight/dose.

19. The composition of claim 16, wherein the fragment of the 39kDa protein is a 28kDa protein and is present in a dosage amount of from about 38 to 3,800 mg/kg of body weight/dose.

20. A t-PA-hepatic clearance-inhibiting protein characterized by binding to low density lipoprotein receptor-related protein (LRP) and inhibiting the binding of t-PA to LRP up to about 80%.

21. A t-PA-hepatic clearance-inhibiting protein comprising modified forms of the 39kDa protein and fragments

```
Pro Arg Leu Glu Lys Leu Trp His Lys Ala Lys Thr Ser Gly Ser Val
 1               5                  10                  15
Arg Leu Thr Ser Cys Ala Arg Val Leu His Lys Glu Lys Ile His Glu
            20                  25                  30
Tyr Asn Val Leu Leu Asp Thr Leu Ser Arg Ala Glu Glu Gly Tyr Glu
        35                  40                  45
Asn Leu Leu Ser Pro Ser Asp Met Thr His Ile Lys Ser Asp Thr Leu
    50                  55                  60
Ala Ser Lys His Ser Glu Leu Lys Asp Arg Leu Arg Ser Ile Asn Gln
65                  70                  75                  80
Gly Leu Asp Arg Leu Arg Lys Val Ser His Gln Leu Arg Pro Ala Thr
                85                  90                  95
Glu Phe Glu Glu Pro Arg Val Ile Asp Leu Trp Asp Leu Ala Gln Ser
            100                 105                 110
Ala Asn Phe Thr Glu Lys Glu Leu Glu Ser Phe Arg Glu Glu Leu Lys
        115                 120                 125
His Phe Glu Ala Lys Ile Glu Lys His Asn His Tyr Gln Lys Gln Leu
    130                 135                 140
Glu Ile Ser His Gln Lys Leu Lys His Val Glu Ser Ile Gly Asp Pro
145                 150                 155                 160
Glu His Ile Ser Arg Asn Lys Glu Lys Tyr Val Leu Leu Glu Glu Lys
                165                 170                 175
Thr Lys Glu Leu Gly Tyr Lys Val Lys Lys His Leu Gln Asp Leu Ser
            180                 185                 190
Ser Arg Val Ser Arg Ala Arg His Asn Glu Leu
        195                 200
```

FIG. 2

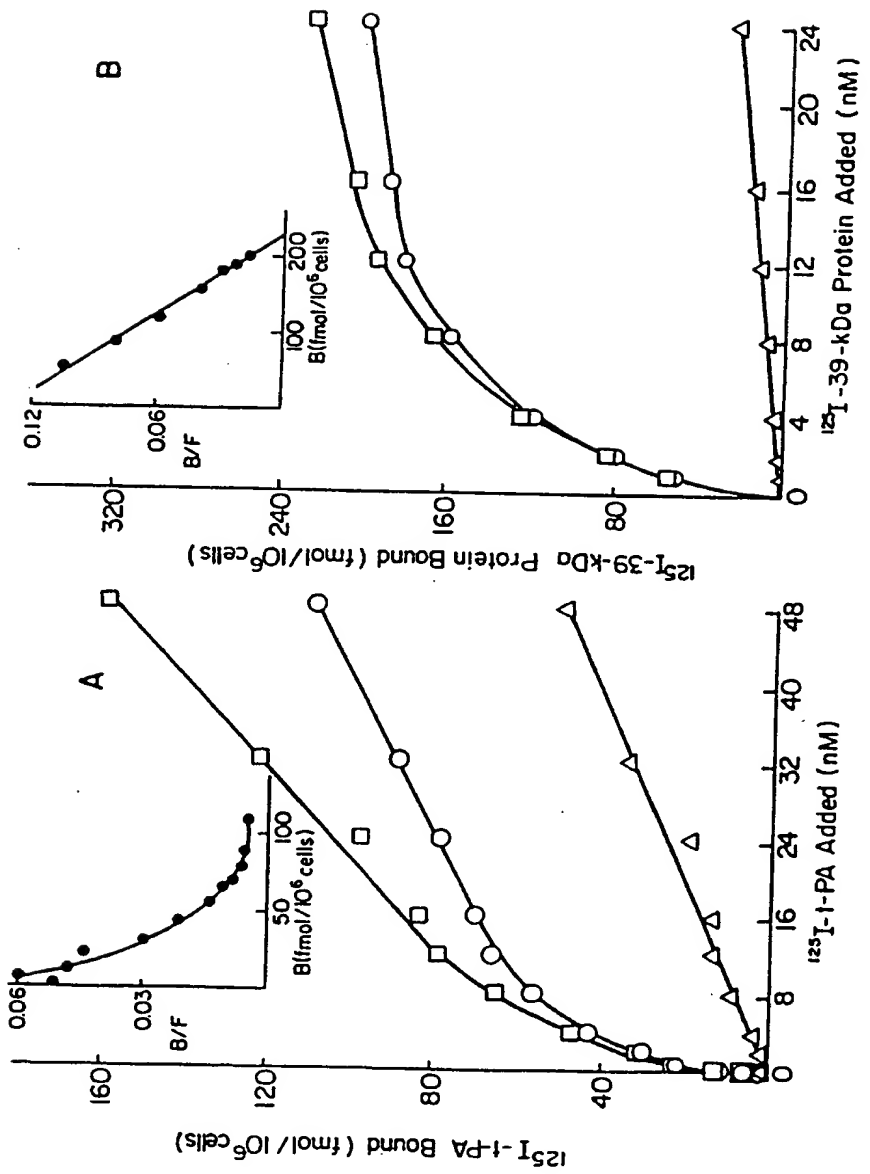
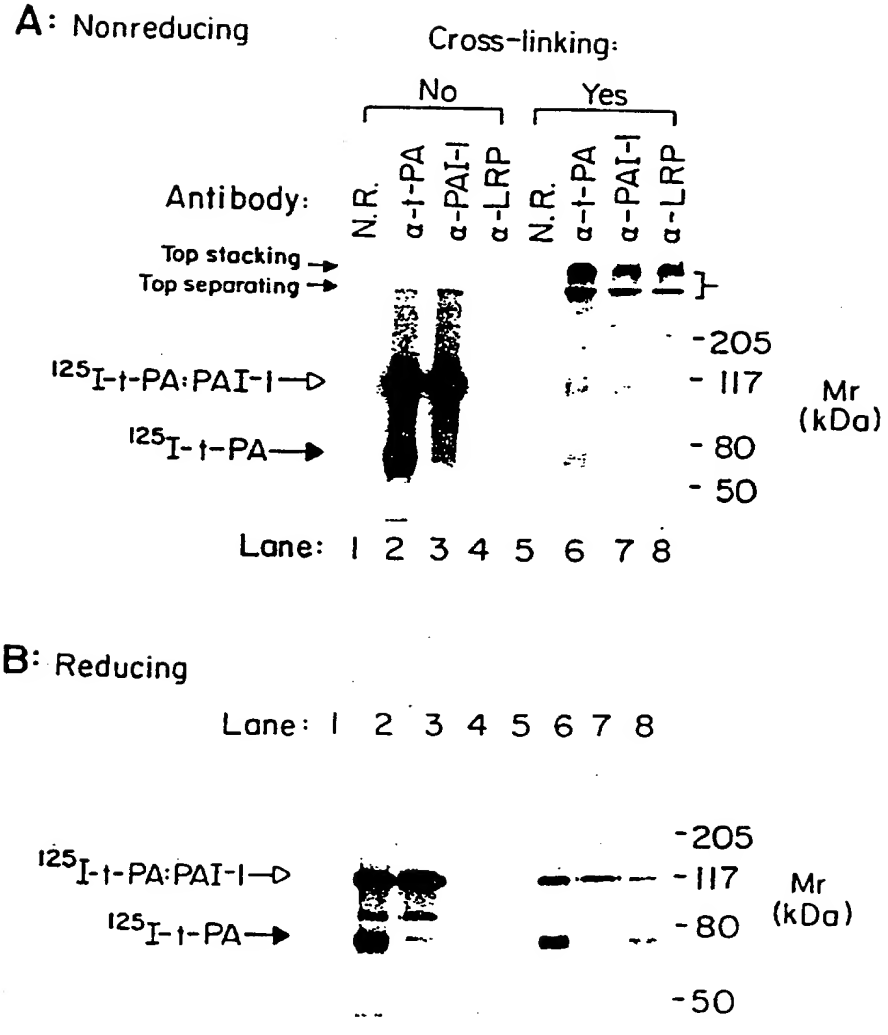

A
B
^{125}I-1-PA Bound (fmol/ 10^6cells)
^{125}I-1-PA Added (nM)
B/F
B(fmol/10^6cells)
^{125}I-39-kDa Protein Bound (fmol/10^6cells)
^{125}I-39-kDa Protein Added (nM)

FIG. 4


A: Nonreducing
Cross-linking:
No
Yes
Antibody:
N.R.
α-t-PA
α-PAI-1
α-LRP
N.R.
α-t-PA
α-PAI-1
α-LRP
Top stacking
Top separating
125I-t-PA:PAI-1
125I-t-PA
-205
-117
-80
-50
Mr (kDa)
Lane: 1 2 3 4 5 6 7 8
B: Reducing
Lane: 1 2 3 4 5 6 7 8
125I-t-PA:PAI-1
125I-t-PA
-205
-117
-80
-50
Mr (kDa)

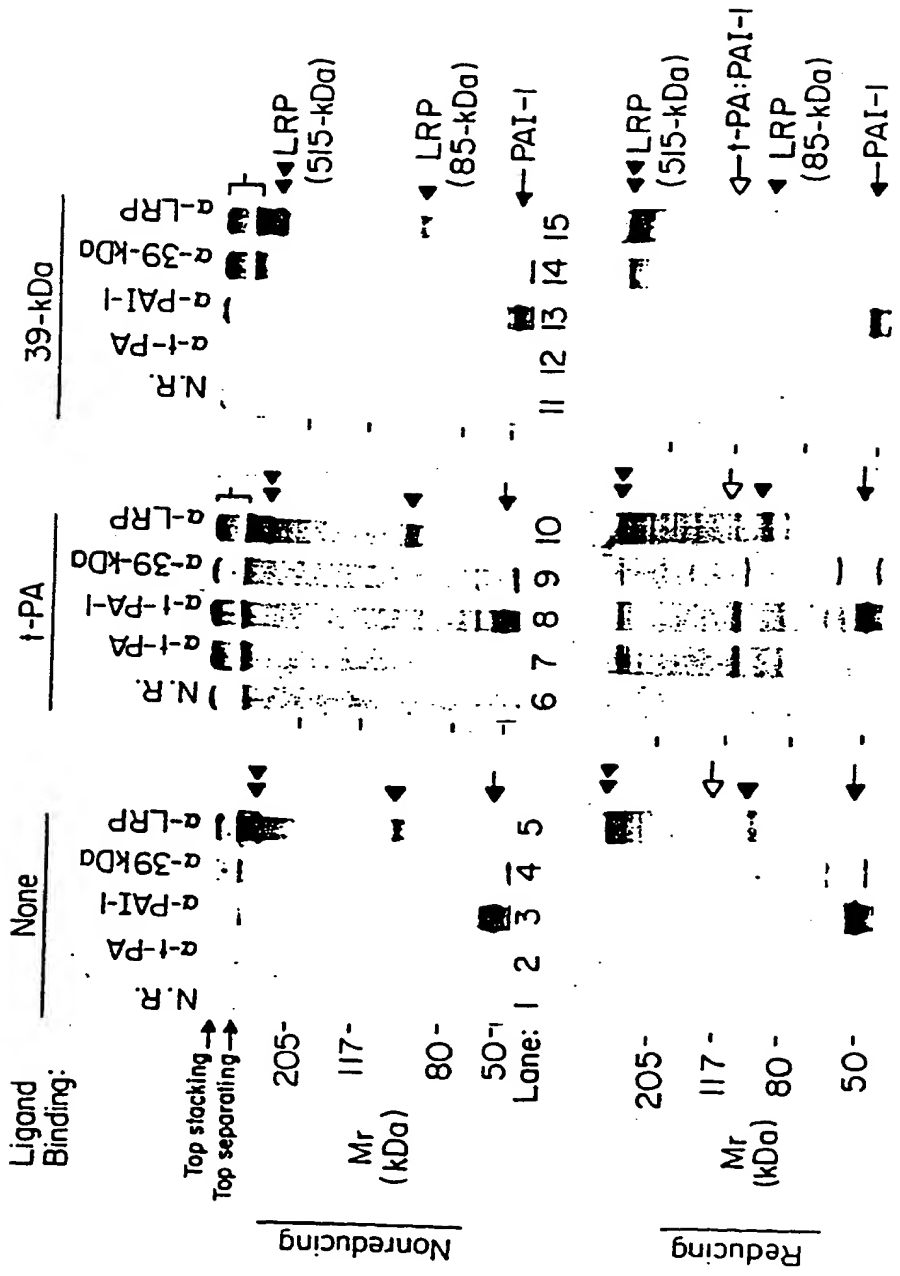

Ligand Binding:
None
N.R.
α-1-PA
α-PAI-1
α-39kDa
α-LRP
t-PA
N.R.
α-1-PA
α-1-PA-I
α-39-kDa
α-LRP
39-kDa
N.R.
α-1-PA
α-PAI-1
α-39-kDa
α-LRP
Top stacking
Top separating
Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Mr (kDa)
205-
117-
80-
50-
LRP (515-kDa)
LRP (85-kDa)
PAI-1
t-PA:PAI-1
Nonreducing
Reducing

FIG. 6

A.
crosslinked
non-reduced
excess unlabelled 39 kDa:
200-
97.4-
69-
46-
kDa
1 2 3 4 5 6 7 8
B.
crosslinked
reduced
excess unlabelled 39 kDa:
200-
97.4-
69-
46-
kDa
1 2 3 4 5 6 7 8
N.R.
α-LRP
α-39kDa
α-I-PA
C.
non-crosslinked
non-reduced
excess unlabelled 39kDa:
200-
97.4-
69-
46-
kDa
1 2 3 4 5 6 7 8
D.
non-crosslinked
reduced
excess unlabelled 39 kDa:
200-
97.4-
69-
46-
kDa
1 2 3 4 5 6 7 8
N.R.
α-LRP
α-39kDa
α-I-PA
E.
[125I]-39kDa, only
crosslinked, non-reduced
excess unlabelled 39kDa:
top stacking
top resolving
200-
97.4-
69-
46-
kDa
39 kDa
1 2 3 4 5 6 7 8
F.
[125I]-39 kDa, only
crosslinked, reduced
excess unlabelled 39kDa:
top stacking
top resolving
200-
97.4-
69-
46-
kDa
39 kDa
1 2 3 4 5 6 7 8
N.R.
α-LRP
α-39kDa
α-I-PA
N.R.
α-LRP
α-39kDa
α-I-PA

FIG. 8

FIG. 10

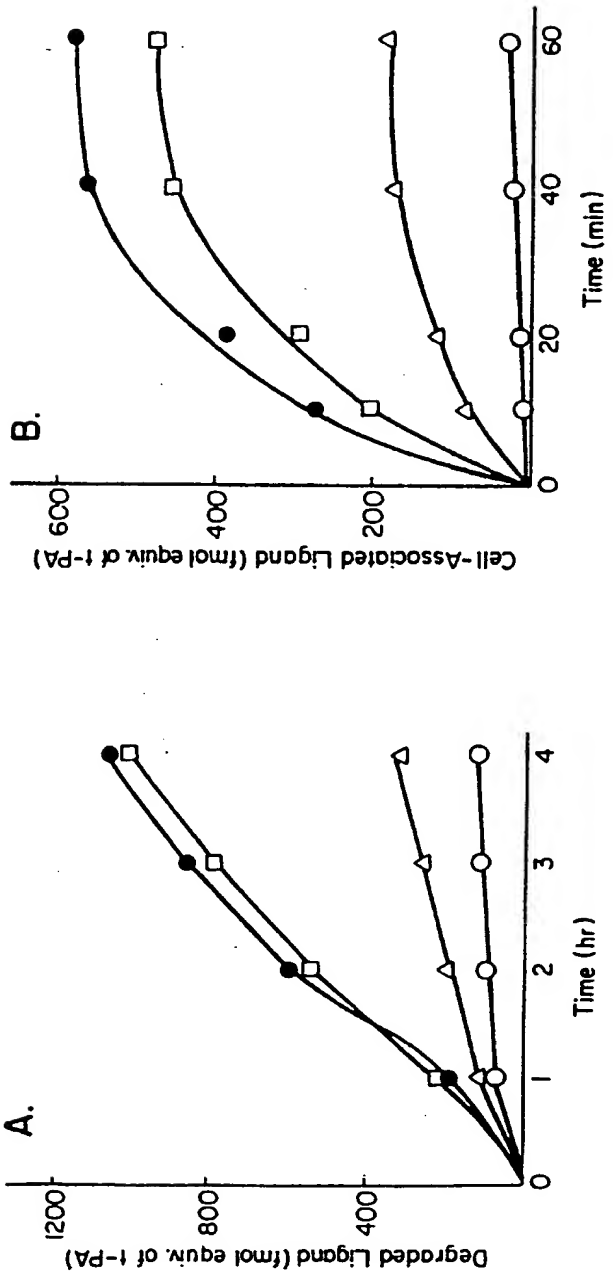
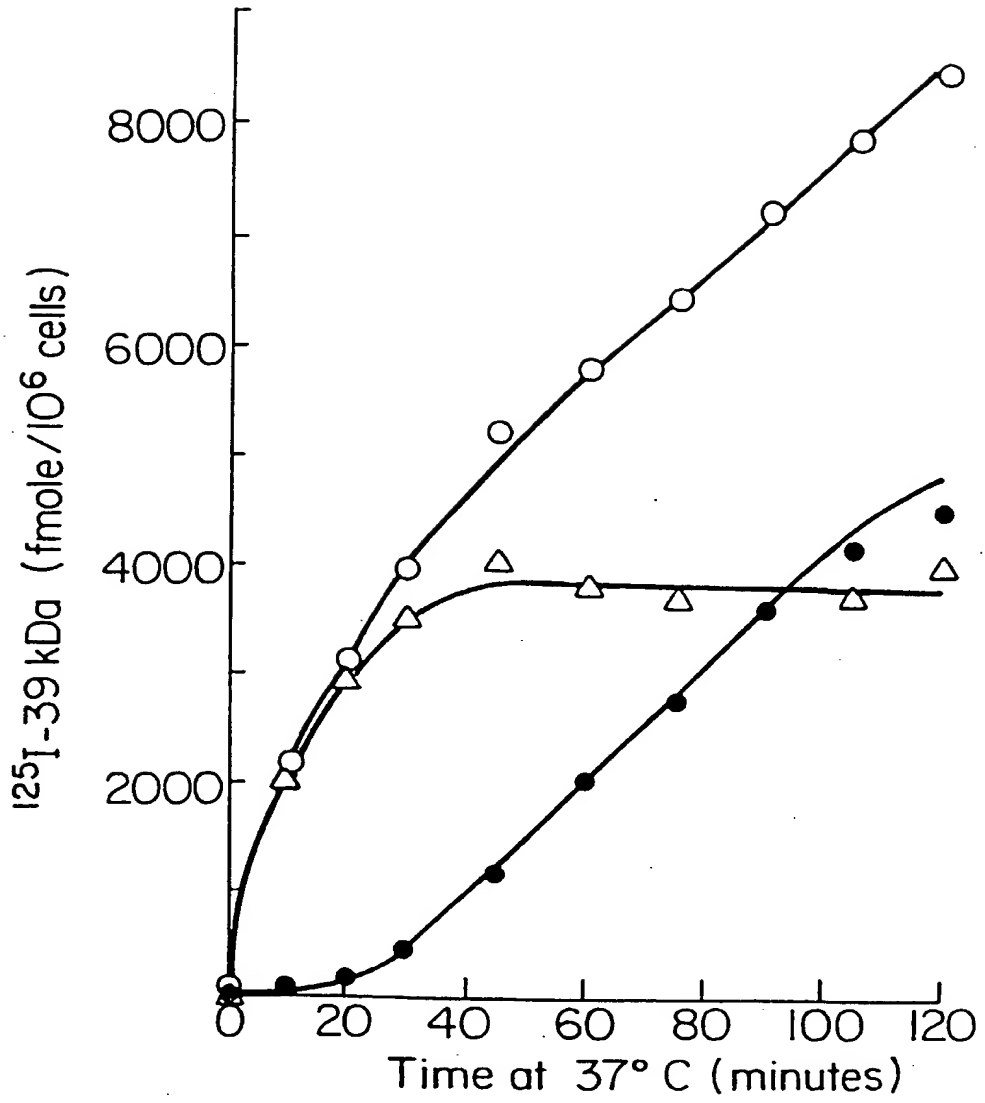

A.
Degraded Ligand (fmol equiv. of 1-PA)
1200
800
400
0
1
2
3
4
Time (hr)
B.
Cell-Associated Ligand (fmol equiv. of 1-PA)
600
400
200
0
20
40
60
Time (min)

FIG. 12


8000
6000
4000
2000
^{125}I-39 kDa (fmole/10^6 cells)
0
20
40
60
80
100
120
Time at 37° C (minutes)

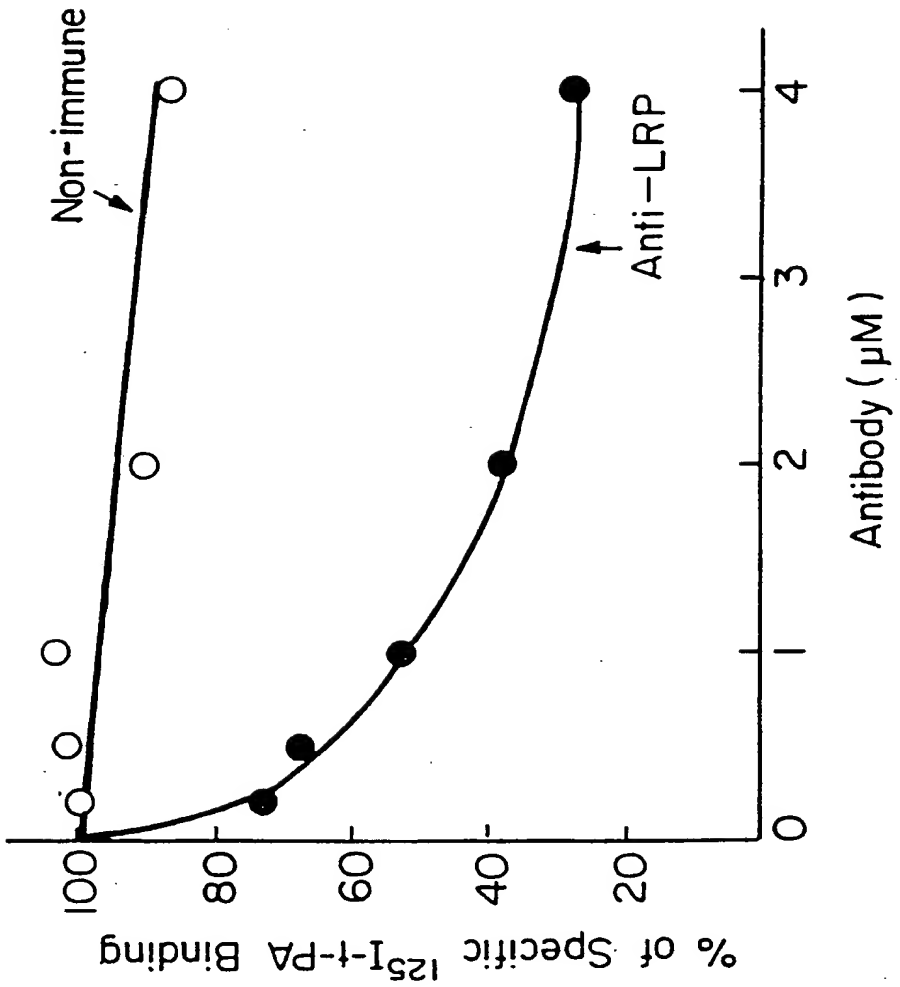

% of Specific ^{125}I-t-PA Binding
100
80
60
40
20
0
1
2
3
4
Antibody (μM)
Non-immune
Anti-LRP

FIG. 14

FIG. 16

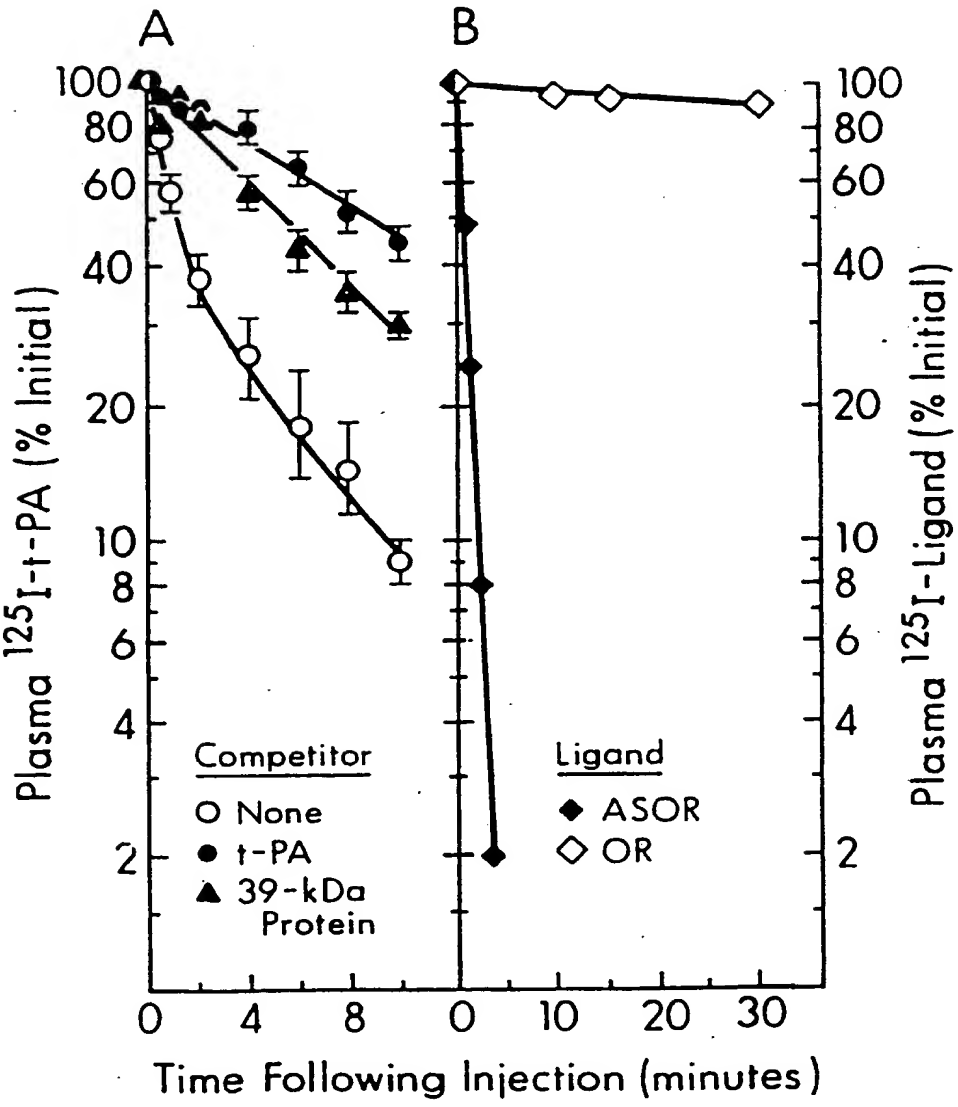
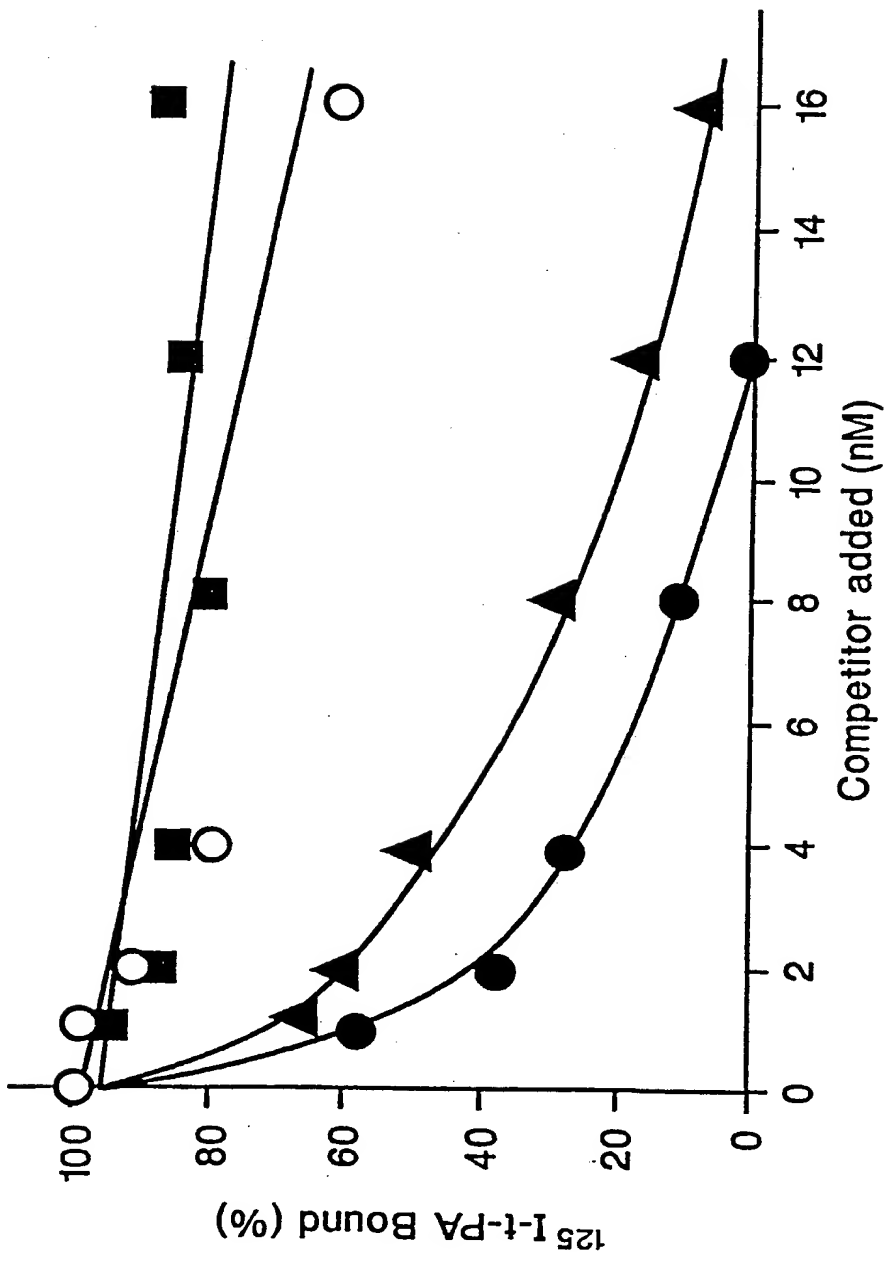

A
B
100
80
60
40
20
10
8
6
4
2
Plasma ^{125}I-t-PA (% Initial)
Plasma ^{125}I-Ligand (% Initial)
Competitor
O None
● t-PA
▲ 39-kDa Protein
Ligand
◆ ASOR
◇ OR
0
4
8
0
10
20
30
Time Following Injection (minutes)

FIG. 18

Inhibition of ^{125}I-t-PA Binding to Rat Liver MH_1C_1 Cells by 39 kDa Protein, 20 kDa N-, and 28 kDa C-terminal Fragment



C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	The American Society of Biochemistry and Molecular Biology, Baltimore J. HERZ et al. "39-kDa Protein Modulates Binding of Ligands to Low Density Lipoprotein Receptor-related Protein/alpha 2 - Macroglobulin Receptor", pages 21232-21238, abstract. --	
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 263, no. 22, issued 1988, August 05 The American Society of Biochemistry and Molecular Biology, Baltimore D.A. OWENSBY et al. "Receptor-mediated Endocytosis of Tissue-type Plasminogen Activator by the Human Hepatoma Cell Line Hep G2", pages 10587-10594, abstract. --	1
A	THROMBOSIS AND HAEMOSTASIS, vol. 54, no. 01, issued 1985, July 14 M. EINARSSON et al. "Endocytosis of Tissue Plasminogen Activator (t-PA) by Rat Liver Cells", page 270, abstract no. P1601. --	1
A	WO, A1, 84/01 960 (BEECHAM GROUP P.L.C.) 24 May 1984 (24.05.84), abstract; page 14, example 5. ----	1

ANHANG

zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

ANNEX

to the International Search Report to the International Patent Application No.

ANNEXE

au rapport de recherche international relatif à la demande de brevet international n°

PCT/US 93/12380 SAE 84484

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben. Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

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Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
WO A1 8401960	24-05-84	EP A1 125269	21-11-84